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PHARMACEUTICAL ANALYSIS



# Rapid Determination of Clonidine in Pharmaceutical Preparations by Paper Spray Tandem Mass Spectrometry (PS-MS/MS)

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**ABSTRACT**

A paper spray tandem mass spectrometric (PS-MS/MS) method without column separation was developed for the rapid screening of clonidine in dietary supplements. PS-MS/MS provided acceptable performance compared to high-performance liquid chromatography – tandem mass spectrometry (HPLC-MS/MS) in terms of limits of detection and quantification, accuracy, and precision. To overcome ionization suppression of complicated plant-based pharmaceutical products, nizatidine, tizanidine, and apraclonidine were investigated as internal standards. A more similar structured internal standard is most suitable for matrix effect compensation. The developed method has advantages of simplicity and speed for the screening of clonidine in dietary supplements.

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## Introduction

Clonidine is a centrally acting α-2 adrenergic agonist and imidazoline receptor agonist (Zhuang et al. 2015). It is a widely used drug to treat hypertensive disorders and hyperactivity disorder (Nirogi et al. 2008). Clonidine has also been used for smoking cessation (Gourlay, Stead, and Benowitz 2004) and management of chronic pain (Kumar et al. 2014; Bamgbade et al. 2022). However, clonidine has side effects such as drowsiness, dry mouth, and postural hypotension. Therefore, the dosage is crucial.

Hypertension is a major cause of stroke, heart disease, and kidney failure (NCD Risk Factor Collaboration (NCD-RisC)), 2021). The market of pharmacotherapy (synthetic drugs) and dietary supplements (including capsules, teabags and traditional Chinese medicines) is enormous. In general, synthetic drugs work quickly but side effects restrict their use (Wang et al. 2009; Zhang et al. 2021). Dietary supplements are helpful for hypertension. However, although the addition of drugs without labels in dietary

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supplements is illegal (Liu et al. 2017a; Chao et al. 2018; Lopez-Garcia et al. 2018), clonidine may be added to antihypertensive dietary supplements (China Food and Drug Administration 2012). To ensure the quality of antihypertensive dietary supplements and protect human health, it is necessary to develop an effective method to determine clonidine in dietary supplements.

Currently, the most common analytical method for clonidine is liquid chromatography (LC) or LC coupled to tandem mass spectrometry (LC-MS/MS) (Parekh et al. 2008; Marchi et al. 2010; Baharfar et al. 2017; AlRabiah et al. 2020). AlRabiah et al. (2020) used reversed phase C18 column for determination of clonidine in mouse plasma in a pharmacokinetic study. Baharfar et al. (2017) used high performance liquid chromatography with on-chip electromembrane extraction for clonidine in urine and plasma. Parekh et al. (2008) used liquid chromatography – tandem mass spectrometry for clonidine in human plasma with solid-phase extraction. However, these methods are time consuming, require pretreatment, and use a column for the separation. Moreover, it is difficult to screen a large number of samples by high-performance liquid chromatography (HPLC).

Compared with HPLC, ambient ionization mass spectrometry (AIMS) offers fast screening, improved throughput, and widespread applicability (Wang et al. 2012; Yang et al. 2015). Paper spray mass spectrometry (PS-MS) is a kind of AIMS, which is convenient, simple, and has been used for fast qualitative and quantitative analyses (Liu et al. 2010). The sample is added to a triangular paper substrate which is connected to a DC high-voltage power supply to directly ionize the sample components (Wang et al. 2010; Liu et al. 2017b). The ambient ionization facilitates in-situ analysis without pretreatment, making it simple and fast. PS-MS has been used for the analysis of urine, wastewater, and Chinese medicine (Liu et al. 2017c; Han et al. 2018; Zhou et al. 2019; Fedick et al. 2020; Rossini et al. 2020; Min et al. 2021; Yang et al. 2022).

Here is reported a semi-quantitative method for the rapid screening of clonidine in antihypertensive dietary supplements. The matrix effects due to the supplements were characterized. The developed protocol was compared to high performance liquid chromatography – tandem mass spectrometry (HPLC-MS/MS) and demonstrated to provide the simple and rapid determination of clonidine.

## Experimental

### *Materials and chemicals*

The clonidine hydrochloride standard was purchased from the National Institutes for Food and Drug Control (Beijing, China). The nizatidine standard was purchased from Innochem Scientific (Beijing, China). The tizanidine standard was purchased from Leyan Scientific (Shanghai, China). The apraclonidine standard was purchased from MedChemExpress (Shanghai, China).

Pharmaceutical samples were purchased from Laobaixing Pharmacy, Qianjin Pharmacy, Dajiaweikang Pharmacy (Changsha, China), and online shops. These samples included dietary supplements (capsules, teabags, and Chinese traditional medicines) for hypertension, Zhenju Jiangya tablets (clonidine compounding drug), clonidine, and clonidine transdermal patches.

HPLC-grade methanol (MeOH) was purchased from Innochem (Beijing, China). HPLC-grade formic acid was purchased from Kermel Chemical Reagent (Tianjin, China). Chromatography paper (Whatman 1) was purchased from J & K Scientific (Beijing, China).

### PS-MS/MS analysis

PS-MS/MS was performed using an 8040 triple quadrupole mass spectrometer (Shimadzu, Japan) combined with a custom paper spray setup. A three-dimensional translational stage was constructed to adjust the position of the PS tip in front of the MS cone. The tip of the paper was placed approximately 5 mm from the inlet.

The analytes were detected in the positive ionization mode. The temperatures were at 250 °C and 300 °C for desolvation and heating, respectively. The full scan mass spectra were acquired from *m/z* 100 to 400. The multiple reaction monitoring (MRM) mode was used for analysis (Table S1).

The spray voltage (+3.0 kV) was applied to the paper triangle using an alligator clamp. 20 µL of the sample with internal standard were added to the paper triangle to generate the electrospray. PS-MS/MS system control and data acquisition were performed by the LabSolutions workstation (Shimadzu).

### HPLC-MS/MS

HPLC-MS/MS was performed by an 8050 triple quadrupole mass spectrometer (Shimadzu, Japan). The analytes were separated on a Diamonsil C18 column (3 $\mu$m, 150 × 4.6 mm, Dikma Technologies, China) with 0.2% aqueous formic acid (A) and methanol (B) as the mobile phase. The gradient elution was performed as follows. 10% B was the initial mobile phase that was linearly increased to 30% in 3 min, linearly increased to 80% in 6 min, and maintained at 80% B in 0.5 min. B subsequently was linearly decreased to 10% from 6.5 to 7 min and maintained at 10% B up to 12 min (Figure S1). The solvent flow rate was 0.5 mL $min^{-1}$ and the injection volume 3 $\mu$L.

The analytes were detected with electrospray ionization in the positive mode. The temperatures were 250 °C, 240 °C, and 300 °C in the interface, desolvation, and heating block, respectively. The gas flows were 3.0 L $min^{-1}$, 10.0 L $min^{-1}$, and 10.0 L $min^{-1}$ for nebulization, drying, and heating. A capillary voltage of 4 kV was applied.

The full scan mass spectra were acquired from *m/z* 100 to 400 in the multiple reaction monitoring (MRM) mode (Table S1). Instrument control and data acquisition were performed with LabSolutions workstation (Shimadzu).

### Preparation of spray paper tips

Triangles were cut from chromatography paper using a craft cutting plotter (Hefei CNC Equipment, Hefei, China). The paper triangles had a tip angle of 38° and an area of 60 $mm^2$ (base width of 9 mm, height of 13.2 mm). The paper triangles (5.5 g) were washed with methanol for 30 min in an ultrasonic bath at room temperature. The

methanol was removed, the paper triangles were placed on a clean glass plate, dried overnight in the fume hood, and subsequently stored in a clean plastic bottle.

## Preparation of standards

1000 $\mu$g mL$^{-1}$ stock solutions of clonidine and nizatidine were prepared in methanol. The clonidine working solutions for PS-MS/MS and HPLC-MS/MS analysis were 0.001, 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, and 5 $\mu$g mL$^{-1}$. The concentration of the nizatidine internal standard (IS) was 0.5 $\mu$g mL$^{-1}$. All stock solutions were stored in a refrigerator and brought to room temperature before use.

## Preparation of samples

One gram of pharmaceutical sample was extracted with methanol (10 mL) for 30 min with sonication. The capsule shell was removed before extraction. The tablets were pulverized before extraction. The extracts were centrifuged at 12,000 rpm for 5 min. The supernatant was filtered before analysis.

## Evaluation of matrix effect (de Sousa et al. 2012)

In order to evaluate the influence of matrix components in mass spectrometry, 5 $\mu$mol L$^{-1}$ clonidine in methanol and in 13 dietary supplement extracts solvent were analyzed. The samples included 3 teabags, 4 capsules, and 6 Chinese traditional medicines is shown in Table 1. The same series of solutions were prepared with nizatidine, tizanidine, and apraclonidine that were employed for the internal standard comparison.

**Table 1.** Ingredients in the dietary supplements, the values of matrix effects upon clonidine, and classification into the matrix mixtures.

| Number | Sample | Ingredients | Matrix effect | Matrix mixture |
|---|---|---|---|---|
| 1 | Teabag | Prunellae spica, cassiae semen, leonuri fructus, uncariae ramulus cum uncis, scutellariae radix, tea | 4.93% | a |
| 2 | Tablet | Eucommiae cortex, scutellariae radix, uncariae ramulus cum uncis, prunellae spica, leonur iherba | 2.86% | a |
| 3 | Tablet | Notoginseng total saponins | 4.02% | a |
| 4 | Capsule | Fish oil (timnodonic acid, docosahexaenoic acid) | 150.94% | b |
| 5 | Capsule | Concentrated phospholipids | 0.80% | c |
| 6 | Capsule | Fish oil (timnodonic acid, docosahexaenoic acid) | 80.62% | b |
| 7 | Capsule | Phospholipids, soybean oil, gelatin, glycerol, purity water | 1.33% | c |
| 8 | Teabag | Spocyni veneti folium, *Apocymum venetum* flower, green tea | 44.63% | d |
| 9 | Teabag | *Gynostemma pentaphylla* | 8.27% | e |
| 10 | Teabag | *Apocymum venetum* | 76.76% | d |
| 11 | Capsule | *Salviae miltiorrhizae* radix et rhizoma extract, *Puerariae lobatae* radix extract, cassiae semen extract, eucommiae cortex extract, gastrodiae rhizoma extract | 10.72% | e |
| 12 | Tablet | Gastrodiae rhizoma extract, apocyni veneti folium extract, ziziphi spinosae semen extract | 10.80% | e |
| 13 | Teabag | *Apocymum venetum* | 42.86% | d |

The matrix effects (ME %) were evaluated by

$$ME\ \% = \frac{A_{matrix}}{A_{standard}} \times 100\% \quad (1)$$

where $A_{matrix}$ and $A_{standard}$ are peak areas of the analyte in sample extract and in pure solvent. A ME % value of 100% indicates that the ionization of an analyte is unaffected by the matrix. ME % values below and above 100% indicate ionization suppression and enhancement, respectively.

The normalized matrix effect (NME) in the presence of internal standard is described by

$$NME\ = \frac{ME\ \%_{clonidine}}{ME\ \%_{IS}} \quad (2)$$

### Method validation

Matrix solutions were prepared using the extracts from the dietary supplements. Table 1 shows that matrix mixture a consisted of matrices 1, 2 and 3 in equal proportions. Matrix mixture b was composed of matrices 4 and 6 in equal proportions. Matrix mixture c consisted of matrices 5 and 7 in equal proportions. Matrix mixture d was composed of matrices 8, 10, and 13 in equal proportions. Matrix mixture e consisted of matrices 9, 11, and 12 in equal proportions. The optimized internal standard was used and the concentration of clonidine in each matrix mixture was detected in triplicate for the calibration curve.

The limit of detection (LOD) for MS/MS was the signal equal to 3 times the noise and the limit of quantification (LOQ) was10 times the noise. The accuracy of the method was determined by the addition of standards to blanks at low, medium, and high levels. The precision was evaluated using the relative standard deviation (RSD). The samples were analyzed in triplicate.

## Results and discussion

### Clonidine analysis by PS-MS/MS

The interface voltage (2.5, 3.0, 3.5, 4.0, and 4.5 kV) was optimized to obtain the best MS conditions using 0.5 $\mu$g $mL^{-1}$ clonidine (Figure S2). This voltage affects the migration of the solvent and targets on the paper and their ionization efficiency. With an increase in the interface voltage, the ionization efficiency increased. However, above the optimum value, the solvent migration was too fast, and the targets were not fully ionized. 3.0 kV was selected for following measurements.

Linearity was obtained from 0.01 to 1 $\mu$g $mL^{-1}$ with a correlation coefficient of 0.9982. The limits of detection and quantification were 0.003 $\mu$g $mL^{-1}$ and 0.01 $\mu$g $mL^{-1}$, respectively. Considering the significant concentrations of analyte in dietary supplements, the limit of detection is suitable for this application.

All samples were analyzed by PS-MS/MS. Clonidine was detected in the labeled samples. Clonidine was not present in the dietary supplements. Hence, in order to demonstrate suitability for clonidine, the samples were spiked with the analyte as shown in

Figure S3. The results demonstrate that clonidine was detectable in dietary supplements by PS-MS/MS.

### *Matrix effects*

Matrix components degrade the determination of the analytes due to matrix effects and should be considered in the method validation. The value of ME % quantifies the matrix effects for the determination of clonidine in the samples. Figure 1 shows the clonidine signal is suppressed in most samples with the exception of matrix 4. Matrix 4 is fish oil, which contains timnodonic acid and docosahexaenoic acid and enhances the ionization efficiency. The mass spectra of fish oil and fish oil spiked with clonidine are shown in Figure S4. Matrices 8, 10, and 13 show lower suppression values.

Paper spray (PS) is based upon the electrospray ionization (ESI). Hence, the matrix effects in these methods were compared. The matrix effects were similar except in the fish oil. In these samples, enhancement was observed in PS while suppression in ESI. This phenomenon may be caused by high acid concentrations.

Matrix effects cannot be totally eliminated and matrix-matched external standard calibration, internal standard calibration, standard addition calibration, and coupled calibration approaches, are used for compensation (Raposo and Barceló 2021). Internal standard calibration, preferably using stable isotope labeled compounds, is the best option. Although stable isotope labeled compounds are ideal, they are expensive. Thus, a suitable internal standard is necessary for quantitative analysis.

Here, nizatidine, tizanidine, and apraclonidine were employed as the internal standard and the results compared. The structures of clonidine and the internal standards are shown in Figure 2. The structural similarities of nizatidine, tizanidine, and apraclonidine with clonidine are $<$ 60%, 60%, and 90%. The normalized matrix effect value with the

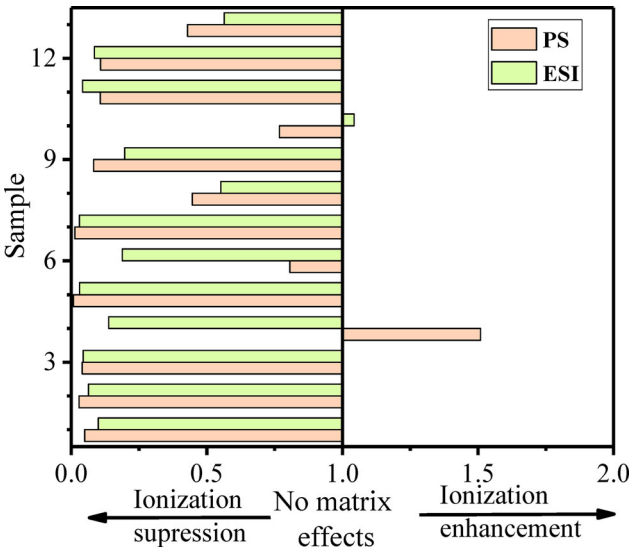


**Figure 1.** Matrix effects for 5 $\mu$mol $L^{-1}$ clonidine in the dietary supplements (ingredients are in Table 1) by paper spray mass spectrometry (PS) and electrospray ionization mass spectrometry (ESI).

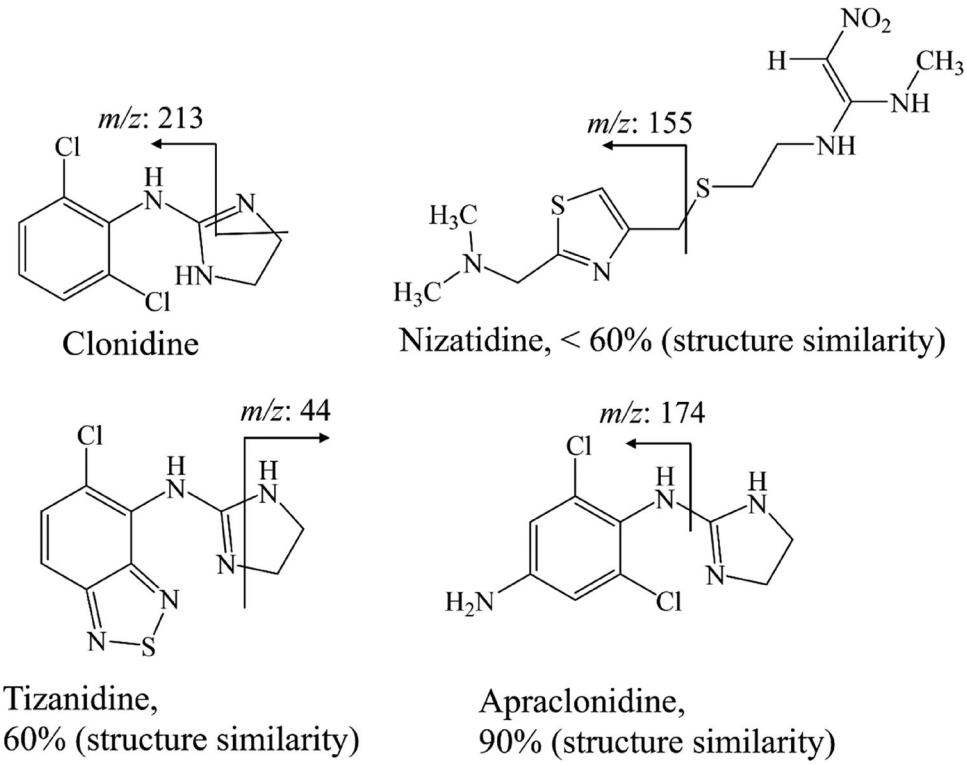


**Figure 2.** Structures and fragmentation of clonidine and the internal standards nizatidine, tizanidine, and apraclonidine.

analyte shows the correction ability of IS. The closer the normalized matrix effect value is to 1, the better the internal standard. Values from 0.8 to 1.2 are acceptable for NME.

Figure 3 shows when nizatidine was used as IS, only 3 NME values are in the ideal range. As the structure similarity increases, the number of NME values in the acceptable range also increases. When apraclonidine is used as IS, most NMF values are between 0.8 to 1.2, except for matrix 4. The results show that the internal standard should be selected based upon the sample type. Hence, tizanidine was employed as the internal standard for matrix 4, and apraclonidine for the other samples.

### *Semi-quantitative analysis by PS-MS/MS*

Five matrix mixtures were prepared for semi-quantitative analysis. Each matrix mixture consisted of 2 to 3 extraction solutions. Table 1 shows that matrix mixture a consists of sample 1, 2, and 3 with matrix effects from 2% to 5%. Matrix mixture b, whose primary ingredient is fish oil, consists of sample 4 and 6 with matrix effects from 80% to 150%. Matrix mixture c, whose primary ingredient is phospholipids, is composed of samples 5 and 7 with matrix effects from 0.1% to 2%. Matrix mixture d, whose primary ingredient is *Apocymum venetum*, includes samples 8, 10, and 13 with matrix effects from 20% to 80%. Matrix mixture e is composed of samples 9, 11, and 12 with matrix effects from 5% to 20%. Based upon the internal standard comparison, tizanidine was used with

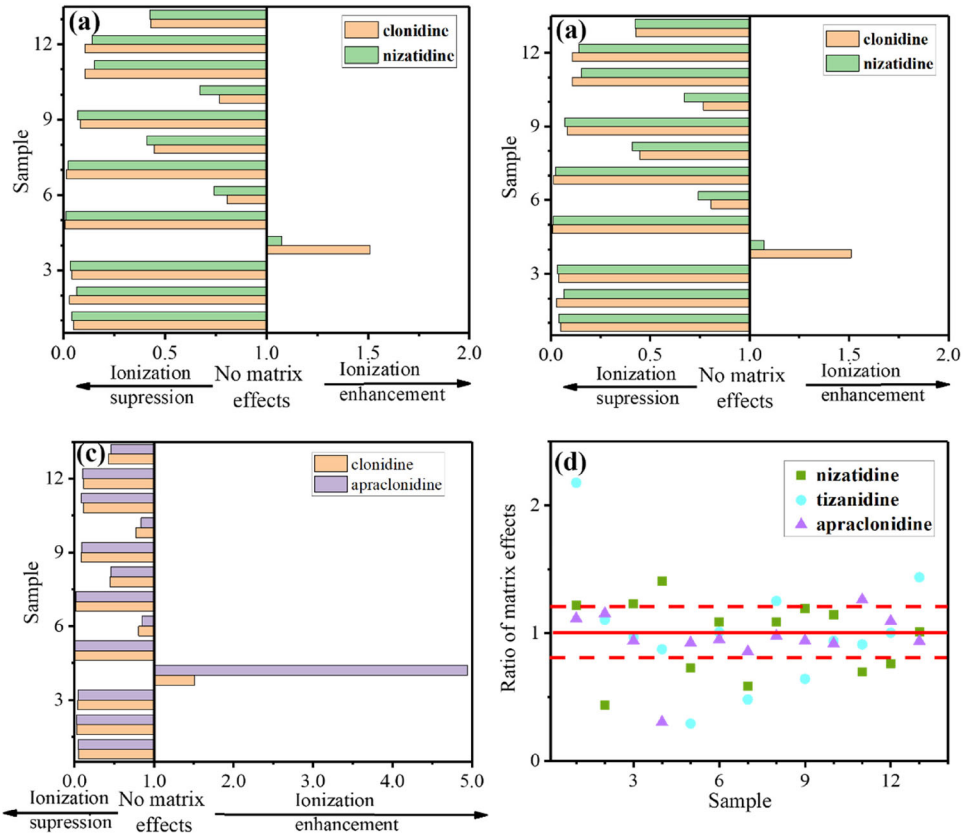


**Figure 3.** Matrix effects of 5 $\mu$mol $L^{-1}$ clonidine and (a) nizatidine, (b) tizanidine, and (c) apraclonidine as the internal standard (IS) in the dietary supplements (ingredients are in Table 1) by paper spray tandem mass spectrometry (PS-MS/MS). (d) Normalized matrix effects for clonidine by nizatidine, tizanidine, and apraclonidine as the internal standard in dietary supplements.

**Table 2.** Analytical figures of merit for clonidine in the matrix mixtures by PS-MS/MS (n = 3).

| Matrix mixture | Regression equation | Correlation coefficient | Linear range ($\mu$g $mL^{-1}$) | Limit of quantification ($\mu$g $mL^{-1}$) |
|---|---|---|---|---|
| a | y = 0.119x + 0.032 | 0.993 | 0.5–40 | 0.5 |
| b | y = 0.059x + 0.002 | 0.999 | 0.05–20 | 0.05 |
| c | y = 0.113x − 0.057 | 0.996 | 2–40 | 2 |
| d | y = 0.064x + 0.005 | 0.999 | 0.2–40 | 0.1 |
| e | y = 0.101x + 0.008 | 0.990 | 0.5–40 | 0.5 |

matrix mixture b and apraclonidine for the others. Table 2 shows even for matrix c, which contains high phospholipids, the limit of quantification is less than or equal to 2 $\mu$g $mL^{-1}$. The developed protocol is suitable for screening purposes with good linearity ($R^2$=0.990–0.999) for each matrix mixture.

HPLC-MS/MS was used as a reference procedure. Table 3 shows that although the standard protocol has a lower limit of quantification (0.005 $\mu$g $mL^{-1}$), PS-MS/MS provides comparable results for the samples. Each HPLC measurement requires 10 min, which is much longer than for PS-MS/MS (< 30 s). The accuracy and precision were

**Table 3.** Clonidine determination in matrix mixtures by PS-MS/MS and HPLC-MS/MS with comparison of the accuracy, precision, and limits of quantitation and detection for PS-MS/MS and HPLC-MS/MS (n = 3).

| | PS-MS/MS | | | HPLC-MS/MS | | |
|---|---|---|---|---|---|---|
| Matrix mixture | Accuracy (%) | Precision (Relative standard deviation) | Limit of detection/Limit of quantitation ($\mu$g mL$^{-1}$) | Accuracy (%) | Precision (Relative standard deviation) | Limit of detection/Limit of quantitation ($\mu$g mL$^{-1}$) |
| a | 81.75–87.48 | 1.58–3.59 | 0.15/0.5 | 97.50–104.3 | 0.28–3.42 | 0.0015/0.005 |
| b | 80.59–101.0 | 3.33–5.75 | 0.015/0.05 | 106.9–110.2 | 0.40–2.41 | 0.0015/0.005 |
| c | 80.87–92.29 | 11.52–20.54 | 0.5/2 | 91.38–93.21 | 0.50–3.03 | 0.0015/0.005 |
| d | 84.89–119.1 | 3.74–4.09 | 0.03/0.1 | 96.23–110.8 | 0.39–0.82 | 0.0015/0.005 |
| e | 81.52–90.39 | 7.09–9.11 | 0.15/0.5 | 91.01–102.3 | 0.35–1.79 | 0.0015/0.005 |

evaluated at 3 concentrations in the linear range. The accuracy of matrix c is degraded, because phospholipids cause severe matrix effects, but it still within the reasonable range. While the accuracy (91.01 to 110.8%) and precision (0.28 to 3.42%) are better for HPLC-MS/MS, the PS-MS/MS method is still quite good, with the accuracy from 80.59 to 119.1% and the precision between 1.58 and 20.54%. Detailed calibration curves are shown in Figures S5 and S6.

## Conclusion

Here is reported a rapid method for the semi-quantitative determination of clonidine in dietary supplements. The limit of detection was 0.003 to 0.5 $\mu$g mL$^{-1}$ in the samples. Good correspondence between the HPLC-MS/MS and PS-MS/MS measurements confirms the applicability of the developed method. A structurally similar internal standard was employed for matrix effect compensation. PS-MS/MS has advantages of simplicity, speed (less than 30 s), and environmentally friendly measurements. Hence this approach is anticipated to be employed for quality control and screening of clonidine.

## Disclosure statement

No potential conflict of interest was reported by the author(s).

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